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Phosphate Solubilizing Activity of Some Bacterial Strains Isolated from Chemical Pesticide Exposed Agriculture Soil

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Abstract*—Phosphorus is one of the most vital macronutrients required for the growth and development of plants. A large number of microorganisms present in the rhizosphere are known to solubilize and make available the insoluble phosphorus in the available form to the plants. A total of fifty phosphate solublizing bacterial colonies were isolated on the Pikovskaya's (PKV) agar medium, containing insoluble tri-calcium phosphate (TCP), from repeatedly chemical pesticide used agriculture soil of Dhanbad area. The colonies showing clear halo zones around the bacterial growth were considered as phosphate solubilizers. Out of 50 bacterial isolates, 10 isolates showing highest phosphate solubilisation index (SI) ranged from 1.4 – 3.0 were selected for the further study as qualitative as well as quantitative performance. Among these 10 potent isolates, two strains S_2 and S_{30} showed the maximum phosphate solubilization index of 3.1 and 3.0 in agar plates along with high soluble phosphate production of 373.07 mgL^{-1} and 368.58 mgL^{-1} in broth culture, respectively. Isolates S_2 and S_{30} belong to genus Bacillus sp. and Pseudomonas sp. as identified by their morphological and biochemical characteristics, respectively. In all the phosphate solubilizing bacterial isolates, decrease in pH was observed ranging 3.2 to 6.2 from initial pH of 6.8 to 7.2. The decrease in pH of the culture medium there by solublizing the insoluble TCP indicated the production of various organic acids by the culture.

***Keywords*—Phosphate solublizing bacteria, pesticide, soil, Bacillus sp., Pseudomonas sp.**

I. INTRODUCTION

Phosphorus is a major essential macro element required for plants to growth and development. The bioavailability of soil inorganic phosphorus in the rhizosphere varies considerably with plant species, nutritional status of soil and ambient soil conditions. It is mostly deficient in soils as it is fixed as water insoluble iron and aluminium phosphates in acidic soils or calcium phosphate in alkaline soils [1]. Chemical phosphate fertilizers are only meagrely soluble under the conditions in which they are applied to the soil. However, under such conditions microorganisms offer a biological rescue capability of solublizing the insoluble inorganic phosphorus of soil. Phosphate solubilizing microorganisms (PSM) particularly those belonging to the genera *Bacillus* sp. and *Pseudomonas* sp., and many others possess the ability to bring insoluble phosphates in soil into soluble forms by secreting organic acids such as formic, acetic, propionic, lactic, glycolic, fumaric and succinic acids [2], [3]. Production of organic acids results in acidification of the microbial cell and its surroundings. These bacteria can grow on various phosphorus containing medium and play an important role in supplying phosphate to plants in a more environmentally-friendly and sustainable manner [4].

Phosphate-solubilizing bacteria (PSB) mobilize insoluble inorganic phosphates from their surrounding soil mineral matrix to the bulk soil where they can be absorbed by plant roots for their growth and development [5]. Although the use of chemical fertilizers is credited with nearly fifty percent increase in agricultural production but they are closely associated with environmental pollution and health hazards [6]. Organic Phosphates applied in agricultural areas do not remain at their target sites, it enter aquatic environments via soil percolation, air drift or surface runoff [7]-[9]. Its concentrations greater than recommended doses in agricultural production do not only cause interruptions in soil microbial activities but they also affect soil environment resulting in alterations in the equilibrium of soil processes for shorter or longer periods. Keeping the above environmental concerns in mind a study being carried out to examine the phosphate solubilization capacity of soil microorganisms present in the chemical pesticide exposed agricultural land and to identify characteristics of most efficient strain using standard microscopic, morphological and biochemical methods.

II. METHODOLOGY

A. Isolation of Phosphate Solubilizing Bacteria (PSB)

A total of 50 Phosphate solublizing bacterial (PSB) colonies were isolated from repeatedly chemical pesticide used agriculture soil from Dhanbad area were isolated on the Pikovskaya's (PKV) agar medium (contained ingredients g/l: Glucose, 10 g; tricalcium phosphate (TCP), 5 g; ammonium sulphate, 0.5 g; sodium chloride, 0.2 g; potassium chloride, 0.2 g; magnesium sulphate, 0.1 g; yeast extract, 0.5 g; manganese sulphate, trace; ferrous sulphate, trace; agar, 15 g; the pH was adjusted to 7.0 ± 0.2 before sterilization [10]) by soil dilution pour plate technique [11]. The bacterial colonies showing phosphate solubilizing zone around them were considered as PSB. Pure culture of the isolates were made by repeated sub culturing for 2-3 times on fresh PKV plate and were maintained on PKV slants at refrigerator temperature.

B. Identification of bacterial strains

Identification of phosphate solublizing bacterial strains was performed by morphological characteristics and biochemical analysis comparing with standard references. The microscopic identification was carried out by gram's staining

method using oil immersion microscope [11]. Morphological and biochemical tests of the PSB isolates were carried out for their identification as per the procedures outlined in Bergey's Manual of Systemic Bacteriology as in [12], [13].

C. Analysis of phosphate solubilizing activity

Out of 50 bacterial isolates, 10 isolates having larger halo zones were selected for further study. The qualitative as well as quantitative analysis of phosphate solubilizing activity of the selected isolates were conducted by plate screening method and broth culture method, respectively. The quantitative analysis of phosphorous solubilizing efficiency was observed by spectrophotometric method at 430 nm.

1). Qualitative measurement of phosphate solubilisation: Bacterial isolates were screened for their tri-calcium phosphate (TCP) solubilizing activity on PKV plates. Isolates were spot inoculated on the centre of agar plate aseptically. All the plates were incubated at 28 ± 2°C for 5-days. A clear zone around a growing colony indicated phosphate solubilisation and was measured as phosphate solubilisation index (SI). SI was calculated as the ratio of the total diameter (colony + halo zone) to the colony diameter [14]. All the observations were recorded in triplicate. Strains developing clear zones around their colonies could easily be identified as PSBs [15], [16].

2). Quantitative measurement of phosphate solubilization: The bacteria, found to be positive for TCP solubilization were further analyzed for their ability to solubilize it in liquid medium. Bacterial isolates were inoculated in Pikovskaya's broth (100 mL) in 250 mL of Erlenmeyer flasks and incubated at 28 ± 2°C for 5 days with interval shaking at 100 rpm. Triplicates were maintained for each treatment. After incubation the bacterial cultures were filtered through Whatmann No.1 filter paper and were clarified by centrifugation at 8,000 rpm for 20 minutes. Uninoculated broth served as control. The soluble phosphorus was determined in clear filtrate using standard procedures as in [17]. The intensity of blue colour was measured in Aquamate Thermo Scientific colorimeter at 430 nm. The pH of filtrate was recorded at the end of the experiment. A standard graph was then prepared from which phosphorus values for experimental samples were calculated.

III. RESULTS AND DISCUSSION

A. Isolation and Identification of Phosphate Solubilizing Bacteria (PSB)

In the present study, the collected soil samples were evaluated *in vitro* for P solubilising bacteria in Pikovskaya's (PKV) plates supplemented with 1.5% (w/v) agar. Initially, 50 isolates were isolated on the basis of zone of clearance around their colonies on PKV plates. Out of 50 bacterial isolates, 10 isolates showed higher phosphate solubilisation index ranged from 1.6 – 3.1 were selected for the further studies. All of the bacterial isolates were rod shaped and 80% of them were gram negative. These isolates were further characterized, by a series of biochemical reactions and identified as genus *Azotobacter* sp., *Bacillus* sp. and *Pseudomonas* sp. (Table I). These bacteria were well known identified as phosphate solubilizer by many researchers [18]-[21].

Table I. Morphological and Biochemical Characteristics of the Isolates

Characteristics	S1	S2	S9	S14	S15	S26	S30	S32	S46	S49
Gram reaction	G -ve rods	G -ve rods	G +ve rods	G -ve rods	G -ve rods	G -ve rods	G +ve rods	G –ve rod	G –ve rod	G -ve rods
Shape	Y/O	Y/O	Y/O	W/T	W/T	W/T	W/T	W/D	Y/O	W/O
Colour										
IMViC test										
Indole	-	-	-	-	+	-	-	-	-	+
Methyl red	-	-	-	-	+	-	-	-	-	+
Vogues Proskauer	-	-	+	-	+	-	+	-	-	+
Citrate utilization	+	+	+	+	+	+	+	+	+	+
H_2S Production	+	+	-	+	-	+	-	+	+	-
Oxidase	+	+	-	+	+	+	-	+	+	+
OF test	+	+	-	+	+	+	-	+	+	+
Nitrate reduction	+	+	+	+	+	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+	+	+	+	+	+
Gelatin hydrolysis	-	-	+	-	-	-	+	-	-	-
Carbohydrate fermentation										
Lactose	-	-	+	-	+	-	+	-	-	+
Dextrose	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+
Mannitol	-	-	+	-	+	-	+	-	-	+
Remark*	P	P	B	P	A	B	B	P	B	A

Y/O- Yellow & Opaque; W/T- White & Translucent; W/D- White & Dry; W/O- White and Opaque.

* A= *Azotobacter* sp., B= *Bacillus sp.* & P= *Pseudomonas sp.*

B. Analysis of phosphate solubilizing activity

1) Quantitative measurement of phosphate solubilisation:

All the selected isolates were found to be potent phosphate solubilizers showing clear halo zone around their colonies. Zone of solubilization around the bacterial colony on PKV agar plates after 3 days of incubation at temperature 28±2°C ranged from 3 to 6.3 mm, the size of the bacterial colony varied from 2.1 to 7 mm. Among these 10 potent isolates, strains S_2 and S_{30} showed the maximum phosphate solubilization activity as visualized by the size of clear zone developed around the colony, which showed solubilization index of 3.1 and 3.0, respectively (Table II). Reference [22] also reported the various zone of solubilization at different incubation temperature ranged from 16.3 (minimum, at 4°C) to 18.5 mm (maximum at 9°C and 21°C), the size of the bacterial colony varied from 4.2 (at 4°C) to 8.8 mm (at 21°C). Reference [23] reported in bacterial isolates the halo size of 2.0 to 5.0 mm on PVK agar and 5.0 to 13.0 mm on MPVK agar. The zone formation could be due to the activity of phosphatase enzyme in bacterial isolates. The experimental PKV slants with phosphate solubilising microbes were stored at 4°C to arrest their growth and activity.

Table II. Phosphate Solubilizing Activities of Ten Most P Solubilising Isolates

Isolates	**Solubilization index†** **(SI)**	**Soluble P concentration (mgL^{-1})**	**Final pH††**
S1 (*Pseudomonas* sp.)	1.8 ± 0.14	308.15 ± 0.82	4.3 ± 0.05
S2 (*Pseudomonas* sp.)	3.2± 0.16	373.07 ± 1.22	3.2 ± 0.08
S9 (*Bacillus* sp.)	1.6 ± 0.07	122.36 ± 0.82	6.2 ± 0.16
S14 (*Pseudomonas* sp.)	1.6 ± 0.16	278.68 ± 1.63	5.4 ± 0.17
S15 (*Azotobacter* sp.)	1.8 ± 0.02	244.39 ± 1.43	5.6 ± 0.09
S26 (*Bacillus* sp.)	1.7 ± 0.02	313.14 ± 2.04	4.6 ± 0.26
S30 (*Bacillus* sp.)	2.9 ± 0.16	368.58 ± 0.41	3.5 ± 0.05
S32 (*Pseudomonas* sp.)	1.7 ± 0.07	293.66 ± 1.22	4.8 ± 0.33
S46 (*Bacillus* sp.)	1.7 ± 0.09	306.650 ± 6.52	4.7 ± 0.29
S49 (*Azotobacter* sp.)	1.8 ± 0.02	258.205 ± 1.63	5.7 ± 0.12

Values are mean of three replicates; *SI = (colony diameter + halo zone)/ colony diameter;
± SD ** Initial pH = 7 ± 2.

2) Qualitative measurement of phosphate solubilisation:

The solubilization levels of TCP varied with deferent isolates, all the 10 isolates were capable of solubilizing tri-calcium phosphate (TCP) in broth medium containing 0.5% of TCP. It was observed that 50% strains showed P solubilization activity above 300 mgL^{-1} where as the rest 50% strains were found to less active with maximum activity below 300 mgL^{-1}. In this study, isolate S_2 (*Pseudomonas* sp.) and S_{30} (*Bacillus* sp.) showed highest percent P solubilization when compared to other isolates, by solubilizing 74.6 % and 73.7% of insoluble TCP, respectively (Figure 1). Reference [19] shows two strains of phosphate solubilizing *Pseudomonas aeruginosa* which solubilize P up to 70% under *in vitro* conditions.

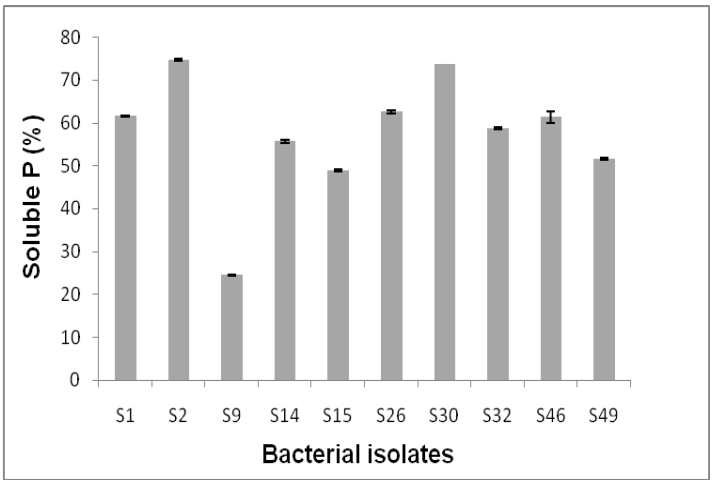

Fig. 1. Solubilization of 0.5% $Ca_3 (PO_4)_2$ by different bacterial isolates.

Strain S_2 (*Pseudomonas* sp.) produced highest soluble phosphate of 373.07 mgL^{-1} followed by S_{30} (*Bacillus sp.*) which produce 368.58 mgL^{-1} of soluble phosphate in the PKV broth (Table II). Our findings are very well supported by the work done by many researchers. Reference [24] shows that PSB isolated from P amended soils solubilized 217–479 mgL^{-1} of P. Moreover reference [25] shows that SBC5 (*Bacillus* sp.) and SBC7 (*Bacillus* sp.) bacterial isolates exhibited maximum P solubilisation of 40 and 33 µg mL^{-1} respectively. Whereas, reference [22] shows the maximum activity of 247 µg mL^{-1} by *Pseudomonas putida*. Reference [26] shows that *Pseudomonas* sp. NBRI 4014 is a potent phosphorus solubilizer (284 g/mL). In the blank treatment no soluble phosphorous was detected and also no drop in pH was observed.

All phosphate solubilizing bacteria assayed showed decrease in the pH of the medium ranged from 3.2 to 6.2 with initial pH 6.8 to 7.2, after 7 days of incubation, which coincided with the increase in the P solubilization activity (Table II). The lowest pH values were scored during the growth phase in which maximal solubilization activity was detected. The maximum drop of pH was observed in S_2 (*Pseudomonas* sp.) (pH =3.2) followed by S_{30} (*Bacillus* sp.) (pH =3.5). In figure 2 and 3, both the strains showed almost similar trend in increase in P solubilisation as the pH decreases. Reference [18] shows that acidification of the broth medium coincided with phosphorus solubilization. Furthermore, Reference [5] also suggested that acidification of culture supernatants can be the main mechanism for P solubilization. It is well known in the literature that PSB solubilise insoluble phosphate in soil by secreting acid, this may indicate that our isolates might have used the same mechanism to solubilize TCP which ultimately caused decline in the pH of culture filtrate. Many of the researchers also suggested that decrease in pH of the culture filtrates containing inorganic phosphate is due to the secretion of organic acids by the bacteria in the culture [27]-[29].

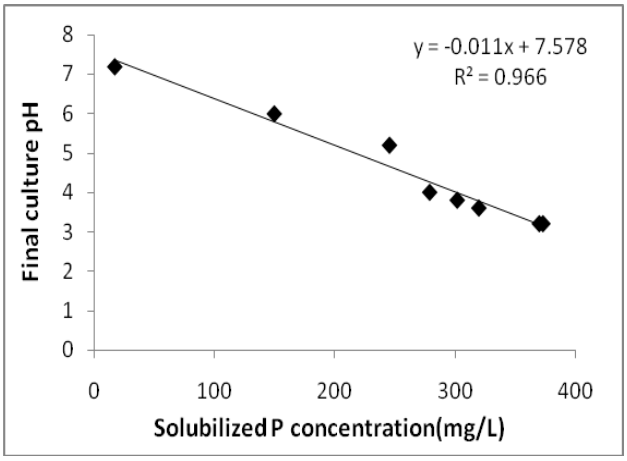

Fig. 2. Relationship between solubilized P concentration and final pH of the culture broth of isolate S_2 (*Pseudomonas* sp.*)*

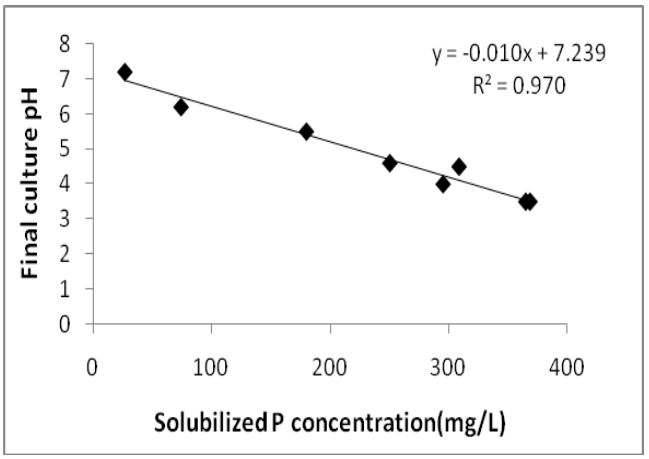

Fig. 3. Relationship between solubilized P concentration and final pH of the culture broth of isolate S_{30} (*Bacillus sp.)*

IV. CONCLUSION

The isolated bacterial strains S_2 and S_{30} (*Bacillus* sp. and *Pseudomonas* sp., respectively*)* are significant phosphate solubilizers. The use of these native strains as bio-fertilizers helps in reducing the use of chemical fertilizers and also effective in reducing the cost of cultivation and maintaining the natural fertility of soil. The decrease in pH of the culture medium there by solublizing the insoluble tri-calcium phosphate indicated the production of multiple organic acids. So, more studies are required to understand the significance and mechanism used by an unknown acid in phosphate solubilization activity. Use of these PSB as bio-inoculants will increase the available P in soil, reduces environmental pollution and promotes sustainable agriculture.

REFERENCES

[1]. S. Singh, and K. K. Kapoor, "Solubilization of insoluble phosphates by bacteria isolated from different sources," *Environ Ecol*, vol. 12, pp. 51–55, 1994.

[2]. M. Rashid, K. Samina, A. Najma, A. Sadia, and L. Farooq, "Organic acids production and phosphate solubilization by phosphate solubilizing microorganisms under *in vitro* conditions," *Pak. J. Biol. Sci*., vol. 7, 187-196, 2004.

[3]. R. Ivanova, D. Bojinova, and K. Nedialkova, "Rock phosphate solubilization by soil bacteria," *Journal of the University of Chemical Technology and Metallurgy*, vol. 41(3), pp. 297-302, 2006.

[4]. M. S. Khan, A. Zaidi, and P. A. Wani, "Role of phosphate-solubilizing microorganisms in sustainable agriculture - A review," *Agron. Sustain. Dev*., Vol. 27, pp. 29-43, 2007.

[5]. E. Pe´rez, M. Sulbara´n, M. M. Ball, and L. A. Yarza´bal, "Isolation and characterization of mineral phosphate-solubilizing bacteria naturally colonizing a limonitic crust in the south-eastern Venezuelan region,' *Soil Biology & Biochemistry*, vol. 39, pp. 2905–2914, 2007.

[6]. A. C. Gaur, and S. Gaind, Phosphate solubilizing microorganisms-An overview. In *Agromicrobes*, Current trends in life sciences, Today and tomorrows publishers, New Delhi. 1999.

[7]. B. S. Andersona, and J. W. Hunta, "Integrated assessment of the impacts of agricultural drain water in the Salinas River (California, USA)," *Environmental Pollution*, vol. 124(3), pp. 523–532, 2003.

[8]. M. G. Rovedatti, "Monitoring of organochlorine and organophosphorus pesticides in the water of the Reconquista River," *Water Research*, vol. 35(14), pp. 3457–3461, 2001.

[9]. W. N. Sawaya, A. Fawzia, and A. Awadhi, "Dietary intake of organophosphate pesticides in Kuwait," *Food Chemistry*, vol. 69(8), pp. 331–338, 2000.

[10]. R. I. Pikovskaya, "Mobilization of phosphates in soil in connection with the vital activities of some microbial species," *Mikrobiologiya*, vol. 17, pp. 362-370, 1948.

[11]. K. R. Aneja, *Experiments in Microbiology Plant Pathology, Tissue Culture and Mushroom production Technology*, 3rd edn. New Age International Publishers, 2002.

[12]. N. R. Krieg, and J. G. Holt, *Bergey's Manual of Systematic Bacteriology*, Williams and Wilkins, Baltimore, U.S.A., 1984.

[13]. P. H. A. Sneath, N. S. Mair, M. Elisabeth Sharpe, and J. G. Holt, *Bergey's Manual of Systematic Bacteriology*, Williams and Wilkins, Baltimore, U.S.A., 1986.

[14]. M. Edi Premono, A. M. Moawad, and P. L. G. Vlek, "Effect of phosphate-solubilizing *Pseudomonas putida* on the growth of maize and its survival in the rhizosphere," *Indones J Crop Sci*, vol. 11, pp. 13–23, 1996.

[15]. P. Gyaneshwar, L. J. Parekh, G. Archana, P. S. Poole, M. D. Collins, R. A. Hutson, and N. Kumar G, "Involvement of a phosphate starvation inducible glucose dehydrogenase in soil phosphate solubilization by *Enterobacter asburiae," FEMS Microbiol. Lett.*, vol. 171, pp. 223–229, 1999.

[16]. W. V. B. Sundara-Rao, and M. K. Sinha, "Phosphate dissolving micro-organisms in the soil and rhizosphere," *Indian J Agric Sci*, vol. 33, pp. 272–278, 1963.

[17]. N. S. Subba Rao, Phosphate solubilization by soil microorganisms. In *Advances in Agricultural Microbiology*, Oxford and IBH Publishing Co, New Delhi, 1982.

[18]. M. M. Collavino, P. A. Sansberro, L. A. Mroginski, and O. M. Aguilar, "Comparison of *in vitro* solubilization activity of diverse phosphate-solubilizing bacteria native to acid soil and their ability to promote *Phaseolus vulgaris* growth," *Biol Fertil Soils,* vol. 46, pp. 727–738, 2010.

[19]. D. Kothamasi, S. Kothamasi, A. Bhattacharyya, R. C. Kuhad, and C. R. Babu, "*Arbuscular mycorrhizae* and phosphate solubilising bacteria of the rhizosphere of the mangrove ecosystem of Great Nicobar island, India," *Biol Fertil Soils,* vol. 42, pp. 358–361, 2006.

[20]. V. Kumar, R. K. Behl, and N. Narula, "Establishment of phosphate-solubilizing strains *of Azotobacter sp. chroococcum* in the rhizosphere and their effect on wheat cultivars under green house conditions," *Microbiol. Res*., vol. 156, pp. 87–93, 2001.

[21]. H. Rodríguez, and R. Fraga, "Phosphate solubilizing bacteria and their role in plant growth promotion," *Biotechnology Advances*, vol. 17, pp. 319–339, 1999.

[22]. A. Pandey, P. Trivedi, B. Kumar, and L. M. S. Palni, "Characterization of a phosphate solubilizing and antagonistic Strain of *Pseudomonas putida* (B0) isolated from a Sub-Alpine location in the Indian Central Himalaya," *Current microbiology*, vol. 53, pp. 102–107, 2006.

[23]. R. Gupta, R. Singal, A. Shanker, R. C. Kuhad, R. K. Saxena, "A modified plate assay for screening phosphate-solubilizing microorganisms," *J Gen Appl Microbio,l* vol. 40, pp. 255–260, 1994.

[24]. J. H. Park, N. Bolan, M. Megharaj, and R. Naidu, "Isolation of phosphate solubilizing bacteria and their potential for lead immobilization in soil," *Journal of Hazardous Materials*, vol. 185(2–3), pp. 829–836, 2011.

[25]. A. S. Chatli, V. Beri, and B. S. Sidhu, "Isolation and characterisation of phosphate solubilising microorganisms from the cold desert habitat of *Salix alba* Linn. in trans Himalayan region of Himachal Pradesh," *Indian J. Microbiol*, vol. 48, pp. 267–273, 2008.

[26]. A. Gupta, J. M. Meyer, and R. Goel, "Development of Heavy Metal-Resistant Mutants of Phosphate Solubilizing *Pseudomonas* sp. NBRI 4014 and Their Characterization," *Current Microbiology,* vol. 45, pp. 323–327, 2002.

[27]. P. Vyas, P. Rahi, A. Chauhan, and A. Gulati, "Phosphate solubilisation potential and stress tolerance of *Eupenicillium parvum* from tea soil," *Mycol Res,* vol. 111, pp. 931–938, 2007.

[28]. E. Nahas, "Factors determining rock phosphate solubilization by microorganisms isolated from soil," *World J Microbiol Biotechno,l* vol. 12, pp. 567–572, 1996.

[29]. N. Vassilev, M. Vassileva, and I. Nikolaeva, "Simultaneous P solubilizing and biocontrol activity of microorganisms: potentials and future trends," *Appl Microbiol Biotechnol*, vol. 7, pp. 137–144, 2006.